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The phenotype of two additional MMTV-neu N transgenic rats has been determined. Females of both of these lines do not display an abnormal phenotype. In contrast, males of line 6500 develop mammary carcinomas with an average latency of 387 days. This phenotype is highly penetrant, with 77.8% and 100% of the transgenic males carcinoma positive at 14 months and 18 months of age, respectively. Among those rats with carcinomas, the average number of carcinomas/rat was 9.6 and 11.7 for 14 months and 18 months, respectively. Nuclease protection assays have shown that 6500 transgenic females overexpress neu within the mammary gland by approximately 10 fold. This demonstrates that the lack of mammary carcinomas in the 6500 females is not due to a lack of neu overexpression in the mammary gland. Experiments are in progress to investigate the mechanisms underlying the male-specific mammary carcinogenesis in line 6500. In additional experiments, retroviral vectors were used to direct expression of EGFR, erbB3, or erbB4 in the mammary gland of 6500 transgenic and non-transgenic females. None of these retroviral constructs resulted in mammary carcinomas for transgenic or non-transgenic females.

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TABLE OF CONTENTS

INTRODUCTION

The epidermal growth factor receptor (EGFR) family consists of four members. These are EGFR, HER2/erbB2/neu, HER3/erbB3, and HER4/erbB4. All of these genes encode for tyrosine growth factor receptors that play important roles in the growth and differentiation of eukaryotic cells. Of the four, EGFR and HER2/erbB2/neu have been implicated as playing major roles in carcinogenesis of several different tissues, specifically in the mammary gland. (For the remainder of this report, HER2/erbB2/neu will simply be identified as neu). Transgenic mice models have established that overexpression of neu in the mammary gland results in the appearance of mammary carcinomas at a high incidence with a long latency. This demonstrates that transgenic rodent models might be of tremendous value in understanding the roles that neu and the other members of this family play in mammary carcinogenesis. In addition to the mouse, the rat has been widely used as an experimental animal model of mammary carcinogenesis because the rat and human versions of the disease share many characteristics. For this reason, we have created transgenic rats that overexpress neu in the mammary gland. In this report, we describe the spontaneous phenotype of these transgenic rats. Additionally, we have attempted to enhance mammary carcinogenesis in these transgenic rats by overexpression of EGFR, erbB3, or erbB4 selectively within the mammary gland by using retroviral vectors.

BODY

RESULTS

Phenotypes of the MMTV-neu N Transgenic Rats

The phenotypic analysis of lines 6490 and 6500 is nearly complete. 6490 females do not have a phenotype different from non-transgenic rats. Namely, they develop mammary fibroadenomas at a high frequency and mammary carcinomas at a low frequency with advanced age (18-21 months old). 6490 males have been analyzed to date out to 12 months of age without any apparent phenotype. 6500 females also remain tumor free. Virgin transgenic females at 18 months old (n=4) had a mammary carcinoma incidence of 0% at necropsy. Non-transgenic female littermates were similarly carcinoma free. Currently, transgenic females at 12 months old (n=11) are being palpated for mammary tumors. To date, there have been no mammary carcinomas in this group. In contrast to 6500 females, males of this line develop numerous mammary carcinomas at a very high incidence. At 18 months old, transgenic males (n=3) had a mammary carcinoma incidence of 100% with 50% of the glands containing carcinomas for each rat. At 14 months of age, transgenic males (n=9) had a mammary carcinoma incidence of 77.8% with an average of 32.1% of the glands containing carcinomas. Among those rats with carcinomas, the average number of carcinomas/rat was 11.7 and 9.6 for 18 months and 14 months, respectively. These 6500 males had no other pathology, except for one 18 month old male with apparent mammary metastases in the lung, liver, and kidneys. The male mammary carcinomas arise with an average latency of 387 days. All of these 6500 males are heterozygous for the transgene.

Expression Analysis of neu in the Transgenic Female Mammary Gland

In order to determine if neu was overexpressed in the virgin or resting-state mammary gland of transgenic female rats, total RNA was isolated and neu RNA was quantified using a nuclease protection assay (NPA). The expression levels of neu were normalized to 28S rRNA. Compared to non-transgenic females, 4311 females did not overexpress neu either as heterozygotes (n=4) or homozygotes (n=4). Only one 6490 female had marginal neu overexpression (2.7 fold), while the other females did not differ from controls (n=4). In contrast, 6500 females overexpressed neu by an average of 10.3 fold (n=5). Experiments to determine the expression levels in male mammary gland are being planned.

Generation of Homozygous 6490 and 6500 Lines

Attempts to create homozygous 6490 rats were not successful, possibly due to homozygosity being lethal in this line. Currently, efforts are underway to generate homozygous 6500 rats.

Retroviral Expression of EGFR, erbB3, and erbB4 in the Rat Mammary Gland

The human forms of EGFR, erbB3, and erbB4 were previously supplied in the retroviral vector LXSN. In these constructs, the gene of interest is expressed from the retroviral LTR and the selection gene used is neo. These constructs were used to package amphotropic retroviral vectors that were infused into the mammary glands of 6500 transgenic females and their non-transgenic littermates. As a positive control, JR/neu T virus was used, with this virus encoding for the constitutively active mutant of neu. As expected, JR/neu T rapidly led to the induction of mammary carcinomas in both transgenic and non-transgenic females. Upon necropsy 7 weeks post-infusion, 81.8% of the transgenic (n=11) and 66.7% of the non-transgenics (n=15) had mammary carcinomas. In most of the rats, the tumor multiplicity was greater than one. In contrast, neither EGFR, erbB3, or erbB4 were able to induce mammary carcinomas. Six months after infusion, only a few rats contained fibroadenomas which was not related to treatment or restricted to either genotype. Approximately 15 rats of both transgenic and non-transgenic were infused with each experimental viral construct.

DISCUSSION

In this report, we have nearly completed an initial characterization of the spontaneous phenotype of transgenic lines 6490 and 6500. Females of both of these lines do not have any abnormal phenotype. A quantitative nuclease protection assay was used to show that 6500 females do in fact overexpress neu within the mammary gland by approximately 10 fold. The lack of mammary carcinogenesis despite overexpression of neu indicates that neu alone is not sufficient to induce mammary carcinomas in the rat. Unexpectedly, 6500 males develop mammary carcinomas with both a high incidence and multiplicity. Males of lines 4311 and 6490 have not, to date, developed mammary carcinomas. This indicates that line 6500 presents a unique phenotype. This may be due simply to differences in the neu expression levels in the three lines. Although expression levels in the males

have not yet been determined, 6500 males presumably express high levels of neu within the mammary gland as evidenced by the striking phenotype. Because both 6490 and 4311 females have little, if any, neu overexpression, it is plausible that males of these two lines do not have significant neu levels as well. Because of the uniqueness of this phenotype, it cannot be overlooked that the transgene insertion site in line 6500 may play an important role in modulating the mammary carcinogenesis. In any event, the appearance of mammary carcinomas in 6500 males but not females is an interesting and unexpected occurrence. Genetics has clearly established that the transgene is not located on the Y chromosome in line 6500. Therefore, this phenotype strongly suggests that the male hormonal environment is conducive to neu-mediated mammary carcinogenesis. This male phenotype is unique to the neu transgenic rat, and has not been described in the neu transgenic mouse. It is therefore possible that neu-mediated mammary carcinogenesis requires a high androgen environment in the rat, but not the mouse.

In another set of experiments, we addressed the question of whether EFGR, erbB3, or erbB4 would be able to cooperate with neu in rat mammary carcinogenesis. In order to do this, retroviral vectors were used to overexpress the human versions of EFGR, erbB3, or erbB4 in both the 6500 transgenic and non-transgenic mammary gland. We had hypothesized that these proteins would form heterodimers with neu, thereby activating the neu tyrosine kinase activity. It has been well established *in vitro* that such heterodimers form and this is believed to have important implications for cellular growth and differentiation. We had therefore reasoned that co-overexpression of neu and other EGFR family members would enhance mammary carcinogenesis in the neu transgenic rat. However, retroviral infusion of EGFR, erbB3, or erbB4 did not generate mammary carcinomas in either transgenic or non-transgenic rats. There are a few possibilities for why this outcome occurred. The human forms of these genes may have formed ineffective heterodimers with the rat neu that is overexpressed in the transgenics. Alternatively, heterodimers may have failed to form due to low levels of endogenous ligands which are absolutely necessary for dimerization to occur. Alternatively, effective heterodimers may have formed, but the expression of these recombinant genes might have been of a short duration. It is well documented, although the reasons are unclear, that occasionally retroviral vectors *in vivo* show only short-term gene expression. In light of the extremely long latency for mammary carcinogenesis in the 6500 male, it is reasonable that carcinomas induced by heterodimerization of neu and other genes might similarly require a long latency which would not be supported by short-term retroviral expression of the cooperating genes. Finally, the proposed experiments may have failed because heterodimerization by itself is not sufficient for mammary carcinogenesis. Considering the 6500 male phenotype, it is possible that a male hormonal environment is necessary for rat mammary carcinogenesis initiated by the EGFR family of protooncogenes. It should be pointed out that 6500 transgenic females were very susceptible to mammary carcinogenesis initiated by the constitutively active mutant of neu

(neu T). This demonstrates that these transgenics females are not inherently resistant to mammary carcinogenesis and that the neu mutant does not require a male hormonal environment for carcinogenesis.

The findings of this report indicate that it would not be worthwhile to pursue the cooperation of neu and other EGFR family members in rat mammary carcinogenesis. However, the observed phenotype of 6500 transgenic males provides an interesting area of investigation. It is tempting to speculate that neu and male androgens, namely testosterone, cooperate in mammary carcinogenesis. The absence of mammary carcinomas in the transgenic females necropsied to date supports this hypothesis that overexpression of neu is not sufficient for mammary carcinogenesis and may, in fact, require testosterone for full transformation. Therefore, the final year of this predoctoral fellowship will be used to further explore the possible role of androgens in neu-initiated rat mammary carcinogenesis. Based on our preliminary findings, we have applied for and received a National Cancer Institute supplemental grant to support this new research. Please find enclosed a copy of this proposal submitted to the NCI, as well as a modified statement of work that will highlight the major objectives to be accomplished for the final year of this DOD fellowship.

APPENDIX

Major Research Accomplishments

- Line 6500 transgenic males develop mammary carcinomas with a high incidence, high multiplicity and long latency.
- To date, line 6500 transgenic females do not develop mammary carcinomas.
- 6500 transgenic females overexpress neu RNA in the mammary gland by approximately 10 fold compared to non-transgenic rats.
- Infusion of retroviral vectors expressing EGFR, erbB3, and erbB4 into 6500 transgenic and non-transgenic females did not induce mammary carcinomas.

Reportable Outcomes

We have applied for and received funding from the National Cancer Institute in the form of a supplemental grant based on work supported by this award.

STATEMENT OF WORK
(Modified)

Please see the enclosed NCI supplemental grant for a description of the objectives under each Aim.

9/99-2/00: Aims 1a, 1b

2/00-6/00: Aim 2. The short-term goal will be accomplished. The long-term goal will not end until approximately 12/00, which is beyond the funding period from this award.

6/00-7/00: Aim3.1

Research Plan

I. Specific Aims

Over the last several years, we have produced several germline transgenic rats on a Sprague-Dawley (SD) background carrying the wild-type neu (HER-2, erbB2) transgene under the control of the MMTV promoter. The neu expression level of 2 of 3 of our transgenic lines were only slightly above their non-transgenic littermates. One of the 3 lines (line 6500) had a high neu expression level in female mammary gland exceeding their non-transgenic littermates by approximately 10 fold. We are now following several small cohorts of heterozygous rats from this line. The oldest of the cohorts is 12-14 months of age. Four of five male rats of this cohort, which are over 12 months old, have developed mammary carcinomas. Control male SD rats did not develop any mammary carcinomas over this time span. Most male rats from line 6500 with carcinomas had very large numbers of individual carcinomas suggesting that the etiology of these cancers is from the wild type non-mutated neu gene. As will be discussed below, it is very likely that this will be a useful basic and translational model. The following specific aims will further characterize this model and test its usefulness in both the cancer prevention and therapeutic settings. (NOTE: This supplement is specifically linked to the parent grant CA28954 by Aim 4.)

Aim 1. Molecular characterization of our wild-type neu mammary cancer model.

a. Fully sequence the neu transgene in banked mammary carcinomas to test our biological generated hypothesis that only wild type neu exists in these cancers. This sequencing assay will be complemented by a 3T3 transformation assay.

b. Extend transgene expression levels data from our female mammary gland evaluation to multiple organs of both male and female rats including both mammary and prostate (the MMTV promoter is active in the prostate) as well as other organs.

c. Produce additional transgenic lines with the same MMTV-neu construct to evaluate if the integration site modulated either the level of expression or the phenotype. Map all insertion points by FISH analysis.

Aim 2. Biological characterization of our wild-type neu model.

a. Extending this male mammary carcinoma model to female rats.

1. Extend observation time of current cohorts to greater than one year.

2. Breed line 6500 homozygous rats to double the copy number and increase the expression level.

3. Alter the endocrinologic status of the female transgenic rat to increase the testosterone level and/or reduce the estrogen levels.

Aim 3. Hormonal profile.

1. Determine the estrogen, progesterone, and androgen receptor levels of carcinomas of the male and female transgenics.

2. Test carcinoma responsiveness to antiestrogens and antiandrogens.

Aim 4. Modifier gene interaction.

Using both the current male model and the future female model, we will ask if the modifier resistance genes we are identifying and characterizing in the "parent" grant can modulate cancer development in this neu model. We will cross the line 6500 neu transgenics on a Wistar Furth (WF) background to congenic rats carrying the mammary carcinoma dominant resistance gene Mcs-1. We will determine the influence of Mcs-1 on carcinoma number and latency in this F_1 generation.

Aim 5. Evaluate the translational utility of this model in a cancer therapeutic and prevention setting.

1. Therapeutic setting: Define the relationship between neu overexpression level and the efficacy of the newly introduced human therapeutic monoclonal antibody (Herceptin). The neu expression level will be varied genetically by using both heterozygous and homozygous line 6500 transgenics as well as any newly established lines which develop cancer. In addition, we will alter expression levels of neu from its inducible MMTV promoter using a series of modified hormonal supplements.

2. Prevention setting: Determine if the antiestrogen tamoxifen or the monoterpene perillyl alcohol can prevent the formation of neu associated mammary cancers in this transgenic model.

II. Significance

The neu oncogene was first discovered by Robert Weinberg's group from a carcinogen-induced rat neuroblastoma (1). It was activated by a point mutation in its transmembrane domain (2). Later it was shown to be activated in human breast cancer where its overexpression is predictive for a poorer prognosis (3,4). However, in women the activation of this oncogene was due to the overexpression of the unmutated wild-type gene. Activation of neu in women is rarely if ever accomplished by mutation (5). If the mutated neu gene is placed into transgenic mice (6,7) or introduced by retroviral vector into rat mammary gland (8), cancer develops rapidly in females. In contrast, when the wild-type gene is expressed in transgenic mice (9), the majority of mammary cancer develops from the rare cells in which the transgene spontaneously mutates to an activated form in somatic mammary cells (10). It is thus felt that the biology resulting from the mutated and wild-type overexpressed genes may differ. While the mutated neu gene can cause mammary cancers in female mice and rats, it has not been established that the overexpression of neu in human mammary cells participates in breast cancer etiology or, alternatively, is just involved in breast cancer progression.

It is thus important to establish a model in which wild-type neu is overexpressed in the mammary gland. A mouse transgenic mammary model has been established; however, its utility is compromised by the fact that its transgene spontaneously mutates at the extracellular domain to a strongly oncogenic neu in the majority of cancers. In addition, tumors developing in the mouse uniformly lack hormonal responsiveness in contrast to those of rat and human breast cancers. We feel that the rat model that we have developed will overcome these shortcomings of the mouse model.

Beside the need to develop a wild-type neu mammary model to study neu's role in the etiology of breast cancer, there is a need for a wild-type neu hormonally responsive mammary cancer model for translational breast cancer research. In translational cancer research both neu directed and general prevention and therapy translational questions could be addressed with such a model.

An example of a current very important question requiring such a model in which neu is differentially overexpressed in an *in vivo* (male or female) mammary carcinoma is determining the best use of the newly FDA-approved monoclonal antibody (Herceptin) which is directed to the extracellular domain of neu (11-13). Currently, this antibody is used to treat breast cancer in which neu expression has been estimated by qualitative methods to be "highly" expressed. Only limited efficacy is now seen with this drug. It is hypothesized that this drug might work best in a subset of cancers with very high expression levels of neu. This is a difficult question to address solely by the use of clinical trials in that late stage breast cancer presents as a very heterogeneous disease. Thus a model with cancers that controllably overexpress this wild-type receptor is needed for the development of the current antibody therapy in regard to better patient selection. In addition, this model will be key in developing and evaluating alternative neu directed treatments of breast cancer. This will shortly include small molecules which selectively block neu receptor kinase activity as well as neu or toxin conjugated neu antibodies.

We have developed a rat model suitable to address this "Herceptin" question as well as other neu directed therapeutic modeling in which neu is overexpressed in mammary cells under the control of the hormone inducible promoter MMTV. Our current transgenic line 6500 develops mammary cancer and overexpresses neu approximately 10 fold. This level of overexpression can be modified both genetically and hormonally through MMTV promoter activation.

It was unexpected that these mammary cancers first developed in male rats. Interestingly, a large percentage of human male breast cancers have neu overexpression (14,15). It is then very likely that this model will serve an important function in developing treatments for the orphan disease – male breast cancer. The frequency of male breast cancer is approximately 1% of female breast cancer. It has a poorer outcome than breast cancer in females due to a lack of basic and clinical research. It is also likely that this model in the male (or female) will be useful to assess the relationship between the level of neu overexpression and the efficacy of monoclonal antibody breast cancer therapy as described above.

However, it must be asked how in general will this model be used to address female breast cancer. We hypothesize that our model may relate to post-menopausal breast cancer. Epidemiological studies strongly relate elevated serum and urine testosterone levels to an elevated risk of breast cancer in postmenopausal women (16-19). Part of this risk may relate to the conversion of testosterone to estrogen. We hypothesize, however, that the androgen receptor signaling pathway in female breast cancer is also directly involved in the etiology of post-menopausal breast cancer. In this context it should be noted that many breast cancers have androgen receptors (14,20,21). We thus will test if testosterone supplemented female neu transgenic rats will develop mammary cancer. These and other studies outlined here will contribute to a better understanding of the interaction of neu and the androgen signal transduction pathway in breast cancer. Indeed, it has previously been shown that neu can activate the androgen signaling pathway in prostate cancer (22).

III. Preliminary Data

Germline transgenic SD rats were made carrying the rat wild-type neu cDNA under the control of the MMTV long terminal repeat. Three independent transgenic lines were produced; designated 4311, 6490, and 6500. Founder rats and their progeny were determined to be transgenic by PCR, using primers specific for the transgene. In order to determine if neu was overexpressed in the virgin or resting-state mammary gland of transgenic female rats, total RNA was isolated and neu RNA was quantified using a nuclease protection assay (NPA). The expression levels of neu were normalized to 28S rRNA. Compared to non-transgenic SD females, 4311 females did not overexpress neu either as heterozygotes (n=4) or homozygotes (n=4). Only one 6490 female had marginal neu overexpression (2.7 fold), while the other females did not differ from controls (n=4). In contrast, 6500 females overexpressed neu by an average of 10.3

fold (n=5). We now hypothesize high levels of neu expression will be found in the mammary tissue. Experiments to determine the expression levels in male mammary glands are being planned.

Rats from line 6500 have been followed for spontaneous mammary tumor development. To date, five F1 transgenic males and four F1 transgenic females have reached 14 months of age. Four of the males have developed mammary tumors, the first tumors arising at 12.5 months of age. Necropsy and histological analysis on the first affected male rat revealed numerous mammary carcinomas in 10 of the 12 mammary glands. The entire mammary gland was grossly converted to carcinoma, thereby making it impossible to quantify the total tumor number. The pituitary gland was grossly normal, indicating that the formation of the mammary carcinomas was not due to a hyperprolactin-secreting pituitary adenoma. A mammary tumor mass resected from a second male was also confirmed to be composed of numerous carcinomas of the same morphology as seen in the previous case. Histological analysis on several of the carcinoma masses revealed that a single tissue section contained a spectrum of normal epithelium, hyperplastic nodules, and small and large carcinomas. Most of the other affected male rats have multiple palpable carcinomas. Several groups have reported that control male SD rats do not develop mammary carcinomas at ages even greater than two years (23-26). Therefore, the development of mammary carcinomas in line 6500 male rats is clearly due to the presence of the neu transgene. The very high carcinoma burden observed strongly suggests that the etiology of these carcinomas is from the overexpression of the wild-type neu gene, and not from spontaneous somatic activating mutations of the transgene as has been described in the MMTV wild-type neu transgenic mouse (10).

IV. Methods

Aim 1.

a. The techniques to detect both deletion (10) and point mutations (2) of neu have been described. We will essentially follow the same procedures for detecting any possible mutations in the transmembrane domain of neu in mammary carcinomas from transgenic rats. Additionally, we will use a laser capture microscope to aid in the microdissection of individual carcinomas from paraffin embedded sections. If we fail to detect mutations by these techniques, then we will rescue the neu transgene from carcinomas into plasmids and fully sequence the transgene. Sequencing assays will be complemented by transformation assays of NIH 3T3 to test the transforming potential of the carcinoma-derived neu transgene *in vitro*.

b. The expression levels of neu will be compared between age-matched female and male transgenic rats. This will be accomplished by a nuclease protection assay. Expression of neu will be evaluated in both MMTV responsive (mammary gland, prostate, salivary gland) and non-responsive (lung, liver, muscle) tissues.

c. The insertion site of the transgene in all lines developing mammary carcinomas will be initially determined at the subchromosomal level by FISH. The specific insertion site will be determined by gene mapping techniques.

Aim 2.

The hormonal environment of both female and male transgenic rats will be altered by surgical techniques and/or hormonal replacement. Females will undergo bilateral ovariectomy at eight weeks of age. Some ovariectomized females will receive time-release pellet implants of testosterone propionate (TP) (Innovative Research of America). Males will undergo bilateral orchidectomy at eight weeks of age and some castrated males will receive TP pellets. Surgically modified rats will be compared to intact controls. For all experimental categories, transgenic rats

will be compared to non-transgenic littermates. These experiments will have both a short-term and a long-term goal. Some rats from all groups will be sacrificed 17 days after implantation of TP pellets and the expression of neu will be determined in various tissues as outlined under Aim 1. The remainder of the rats will be observed until reaching two years of age, at which time the mammary tumor number and neu expression levels will be determined.

Aim 3.

1. The estrogen, progesterone, and androgen receptor content will be determined in both mammary carcinomas and normal gland. This will be done by a combination of immunohistochemistry and Western blots.

2. The responsiveness of mammary carcinomas arising in transgenic males to antiestrogens and antiandrogens will be determined. When transgenic males develop a mammary carcinoma measuring 7x7 mm, they will be randomized into one of five treatment groups: no treatment, orchidectomy, implantation of tamoxifen time-release pellets (Innovative Research of America), implantation of flutamide pellets (Innovative Research of America), or implantation of both tamoxifen and flutamide pellets. Pellets will be left in the rats for 60 days, during which time mammary carcinomas will be observed for regression.

Aim 4.

The line 6500 neu expressing transgenic line, now on a SD outbred background, will be backcrossed to a WF background. The efficiency of carcinoma development in this line will be determined. It is expected that the tumor yield will be high in that the WF strain is very susceptible to mammary carcinoma development. This WF-neu transgenic will be crossed to the WF Mcs-1 congenic lines bred under the support of CA28954. Mcs-1 is a semidominant mammary carcinoma resistance modifier gene. This gene causes resistance to chemically induced rat mammary cancer. It is very important to ask if this Mcs-1 gene also confers resistance to mammary cancer with a wild-type neu etiology. If positive results are obtained, this will suggest that the human homologue of MCS-1 will be a chemoprevention drug target in humans since it can prevent tumors with neu, an important gene in human breast cancer.

Aim 5.

Male rats and female rats of line 6500 carrying carcinomas will be treated with the neu monoclonal breast cancer drug Herceptin that will be commercially obtained. If this drug is effective in these carcinomas, then its effect in carcinomas with higher or lower levels of neu receptor will be tested for comparison. Chemically induced carcinomas that lack a neu component in their etiology will serve as a control. This will serve as a demonstration of the utility of this model in neu directed therapy. Later, this model will be important in developing other therapies for breast cancers with activated wild-type neu including the anti-neu kinase drugs that are in development.

In the area of prevention, we will first characterize this model for preneoplastic lesions such as DCIS. We expect to see them based on our preliminary histopathological characterization of the carcinomas arising in line 6500. We will then treat these rats with a model class of general breast cancer prevention agents; the monoterpenes (perillyl alcohol) and determine if the number of preneoplastic lesions and cancers developing are reduced.

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